CARDIOREGULATION IN LIMULUS. II. GAMMA AMINOBUTYRIC ACID, ANTAGONISTS AND INHIBITOR NERVES

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The neurogenic beat of the *Limulus* heart has long been regarded the classic example of a neurogenic rhythm (Carlson, 1909). As in other neurogenic hearts, such as those of Crustacea, the rate and strength of beating can be decreased by stimulation of cardioinhibitory nerves arising from the central nervous system (Carlson, 1905; Heinbecker, 1933; Pax and Sanborn, 1964). In *Limulus* the decrease in heart rate is not tightly coupled to stimulation of the inhibitor nerves, a time lag in the response occurring both at the beginning and at the end of the stimulation periods. It is probable, therefore, that inhibition in the *Limulus* heart is chemically mediated (Pax and Sanborn, 1964).

The nature of the chemical mediator of inhibition is not known. 5-Hydroxytryptamine (5-HT, serotonin) has been reported to slow the rate of rhythmic discharge from the isolated cardiac ganglion (Burgen and Kuffler, 1957). However, in other neurogenic hearts, 5-HT and related compounds have excitatory effects (Kerkut and Price, 1964).

Gamma-aminobutyric acid (GABA) has also been reported to inhibit the *Limulus* heart (Burgen and Kuffler, 1957). This compound has inhibitory effects on neuromuscular phenomena in a wide variety of other animals. It is present in lobster inhibitory motor neurons but not in excitatory motor neurons (Kravitz *et al.*, 1963). At the crustacean neuromuscular junction, it mimics the action of the inhibitory transmitter both postsynaptically and presynaptically (Dudel, 1965; Takeuchi and Takeuchi, 1966) and in the crustacean cardiac ganglion GABA closely mimics the action of the inhibitor (Florey, 1957; Maynard, 1961). From this evidence it appears possible that GABA or a GABA-like compound may be responsible for cardioinhibition in the *Limulus* heart. We report here results of experiments exploring this possibility more fully.

A primary requirement of any supposed transmitter is that, when artificially applied, it mimics in all respects stimulation of the prejunctional structure (McLennan, 1963). Stimulation of the cardioinhibitory nerves in *Limulus* results in a decrease in rate and strength of beating of the intact heart, a decrease in the number of units discharging in the cardiac ganglion during each burst of electrical activity and a decrease in the total duration of each burst (Carlson, 1905; Heinbecker, 1933; Pax and Sanborn, 1964). We have tested the ability of exogenously applied GABA to mimic these actions of the inhibitor nerves.

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Since data obtained by application of supposed transmitters to the cardiac ganglion are at best equivocal, we have also followed a second line of investigation. Compounds which block the action of the endogenous transmitter should similarly antagonize the effects of exogenously applied GABA.
this purpose we have used picrotoxin, a compound capable of blocking the action of GABA in other systems (Van der Kloot *et al.*, 1958). We have tested picrotoxin for its ability to block the action of the endogenous transmitter, *i.e.*, block the action of the inhibitor nerves. We have also tested picrotoxin for its ability to block the action of the supposed transmitter artificially applied to the heart. For

Materials and Methods

Source and maintenance of animals

Adult *Limulus polyphemus*, maintained as previously described (Pax and Sanborn, 1964), 20 to 25 cm. maximal width, were used in all experiments. They were shipped by air express at two-week intervals from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts, and maintained in moist excelsior at a temperature of 5° C. Responses of animals so maintained did not vary for at least six weeks.

Animal preparations

Isolation of the heart from *Limulus* requires removal of the tough dorsal exoskeleton. This is best done by sawing through the exoskeleton just lateral to the underlying heart and joining the lateral cuts with transverse anterior and posterior cuts so that a rectangular piece of isolated exoskeleton overlying the heart may be removed by lifting and scraping it free of the underlying tissues. Once this piece of exoskeleton has been removed the internal extensor muscles of the opisthosoma dorsal to the heart in the cephalothorax and the epidermal tissue overlying it in the opisthosoma can be dissected away. The intact heart can then be removed.

Stimulation of inhibitor nerves

As we suggested earlier (Pax and Sanborn, 1964), stimulation of the inhibitor nerves near the ventral nerve ring is undesirable since they also contain fibers which innervate muscles. We have since been able to locate inhibitor fibers as they enter the heart dorsally. At these sites the nerves apparently consist exclusively of cardioinhibitory fibers.

Perfusion of the isolated heart

After removal from the animal the heart was placed in a V-shaped lucite chamber 15 cm. in length. The heart was ligated anteriorly in the second segment and posteriorly a cannula was inserted into the lumen of the heart through the cardiac muscle.

Tension on the heart walls and the amount of intra-lumenal pressure both influence the rate and the strength of beating of the heart (Carlson, 1907). Longitudinal tension approximating that on the heart *in situ* was obtained by stretching the heart to a length equal to that present before removal from the animal.

In order to maintain an intra-lumenal pressure, a gravity-feed reservoir of Chao's (1933) saline solution (0.44 *M* NaCl, 0.009 *M* KCl, 0.037 *M* $CaCl_2$) was connected to the cannula at the posterior of the heart. The hearts were perfused at the rate of 20 ml. per minute, the route of the perfusion fluid being from the lumen of the heart out through the ostia and lateral arteries to the exterior. The total volume of fluid in the chamber was maintained at 10 ml. by providing an overflow in the chamber near the anterior end of the heart.

Recording of data

Electrical activity was recorded from the cardiac ganglion of the intact heart by dissecting it free of the heart muscle in the second and the third segments and placing it over hooked platinum electrodes. From the isolated cardiac ganglion, electrical activity was recorded by stringing the ganglion through a series of 12 platinum loop electrodes spaced five mm. apart. During the course of a single experiment any of these electrodes could be chosen to be used as recording electrode. Measurement of mechanical activity of the heart muscle was obtained with a Statham G10b displacement transducer (maximum displacement 0.15 oz.).

Experimental methods and drugs

All drugs were dissolved in Chao's (1933) saline as shortly before use as practicable. Parallel reservoirs of saline and drug solution were connected to the chamber through a two-channel stopcock so that perfusion could be alternated by a turn of the barrel.

Data reduction

Heart rates in *Limulus* vary greatly from animal to animal (Pax and Sanborn, 1964). Moreover those hearts which have an initial high rate of beating tend to have a greater change in rate during inhibition than do those which have an initial low rate of beating. For these reasons we have, when measuring changes in rate, used each animal as its own control and expressed all rates as relative heart rates. Relative heart rate is defined as the ratio of the experimentally altered rate to the control rate. Thus relative rates of less than one are indicative of inhibition and values greater than one indicate excitation. In a similar manner, all data on strength of contraction of the heart muscle are expressed as relative strengths.

In drug perfusion experiments relative rates and contraction strengths were calculated from the mean rates and contraction strengths during the last two minutes of perfusion. In stimulation experiments relative rates were calculated from the mean rates during the entire time of stimulation.

Results

Gamma-aminobutyric acid

Perfusion of GABA through the intact isolated *Limulus* heart results in a decreased heart rate. A typical result of such perfusion is shown in Figure 1, and the results of 21 such perfusions in 15 different hearts are plotted in Figure 2. The solid line in the figure is the regression line for these data as determined by

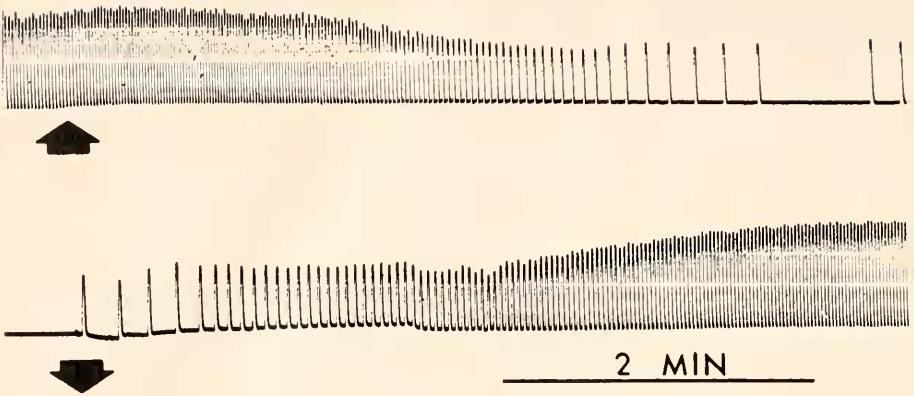

FIGURE 1. The response of the isolated heart to perfusion with GABA. The record is continuous from upper left to lower right. One hundred ml. of 5×10^{-5} *M* GABA were perfused during the time between the two arrows.

the method of least squares; the standard error is indicated by the dashed lines. The slope of the regression line is -0.36, the standard error 0.23. At all concentrations of GABA tested, the rate-slowing effect is readily reversible by perfusion with drug-free saline (Fig. 1).

The strength of contraction of the heart also decreases with GABA perfusion (Fig. 1). In Figure 3 the relationship between relative strength and concentration

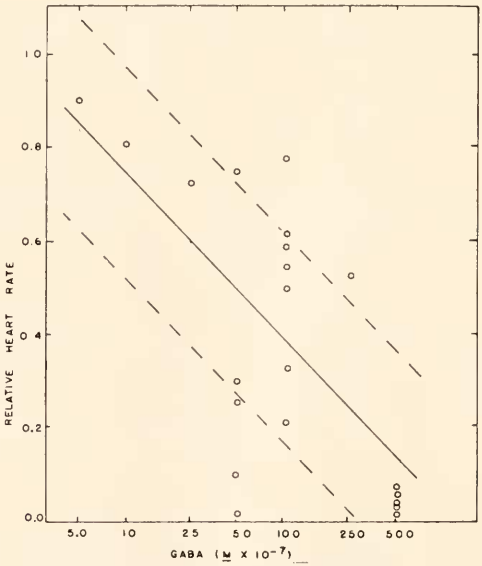

FIGURE 2. Relation of relative heart rate to concentration of perfused GABA. Each point represents a single perfusion. The solid line is the regression line determined by least squares and the dashed lines are the standard error of the regression line.

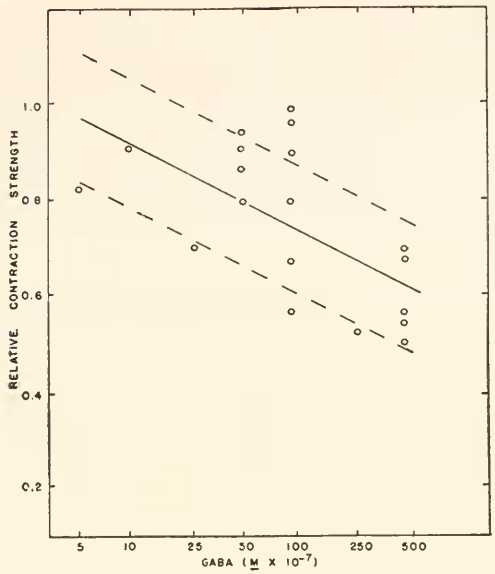

FIGURE 3. Relation of relative contraction strength to concentration of GABA perfused through isolated hearts. The solid line is the regression line determined by least squares and the dashed lines represent the standard error of the regression line.

of perfused GABA is plotted for 19 perfusions in 13 different hearts. The slope of the regression line in this case is only -0.17 compared to the slope of -0.36 for rate changes. Thus a concentration of GABA sufficient to reduce heart rate by 50% reduces contraction strength by less than 20%.

Although GABA reduces the rate at which rhythmic bursts of electrical activity occur in isolated cardiac ganglia, it causes no readily apparent changes in the pattern of the individual bursts. In Figure 4 the pattern of a typical burst of electrical

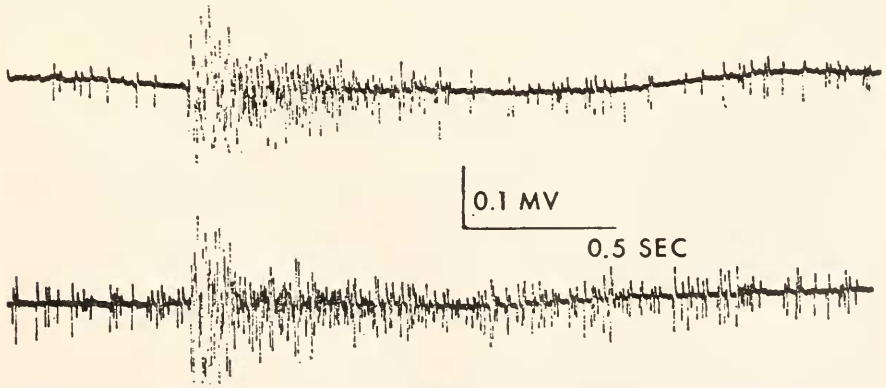

FIGURE 4. The pattern of electrical activity in an isolated ganglion. The upper trace shows a representative burst before drug treatment; the lower trace a representative burst after bathing the ganglion for one minute in 1×10^{-5} *M* GABA.

activity recorded from the fourth segment of an isolated cardiac ganglion in drug-free saline is compared to a typical burst of electrical activity recorded from the same segment of the same isolated cardiac ganglion after bathing in 1×10^{-5} *M* GABA for one minute. At the time of recording the rate of rhythmic bursting has been reduced by 50% but, contrary to the changes seen in the pattern of the burst during stimulation of the inhibitor nerves, there is neither a decrease in the duration of the burst nor a lesser number of discharges in a particular burst. Bathing the ganglion for one minute in drug-free saline is sufficient to return the rate of rhythmic bursting to the pre-treatment level.

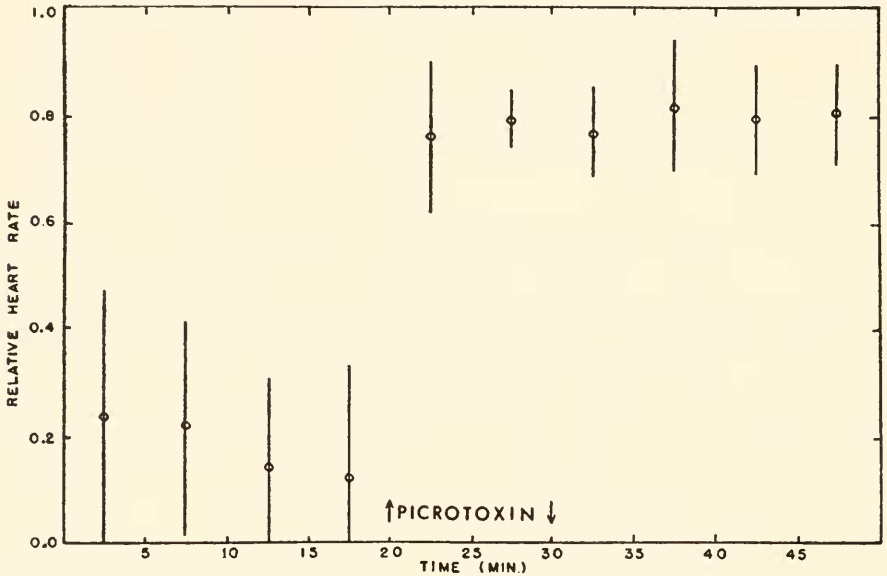

FIGURE 5. Effect of picrotoxin on cardioinhibitory nerves. Each point represents the mean relative heart rate for four hearts during stimulation of the inhibitor nerves. Vertical lines extend one standard deviation on either side of the mean. During the time between the two arrows 100 ml. of 10^{-3} *M* picrotoxin were perfused.

L-glutamic acid at a concentration of 10^{-3} *M* perfused through the isolated heart reduces the strength of contraction of the heart muscle to a barely detectable level but does not change the heart rate. At 10^{-5} *M* it has no measurable effects on rate or strength. In like manner carnitine (gamma-aminobutyric-beta-hydroxy-betaine) perfused through the heart at 10^{-4} *M* causes a marked decrease in strength of contraction but causes no measurable change in rate.

Picrotoxin

The ability of picrotoxin to block the action of the inhibitor nerves was tested in four isolated hearts. In each experiment the inhibitor nerve was stimulated near its junction with the cardiac ganglion in the fourth heart segment. Stimulation was given for 40 seconds out of every five minutes. During the first four such five-

minute intervals drug-free saline was perfused. In the fifth and sixth intervals 100 ml. of 10^{-3} *M* picrotoxin were perfused and then during the next four five-minute intervals drug-free saline was again perfused.

In each case picrotoxin alone caused an increase in heart rate, the mean rate being 24.9 beats per minute before picrotoxin perfusion and 34.6 beats per minute after picrotoxin perfusion. To compensate for this drug-induced rate increase, the relative rates in the portion of the experiment when picrotoxin was used were computed by comparing the ratio of the rate *during* stimulation to that obtained immediately *before* stimulation. Both rates were thus measured in the presence of the drug.

In each of the four hearts the inhibitor nerves were less effective during picrotoxin perfusion. In two of these this decreased effectiveness preceded the increase

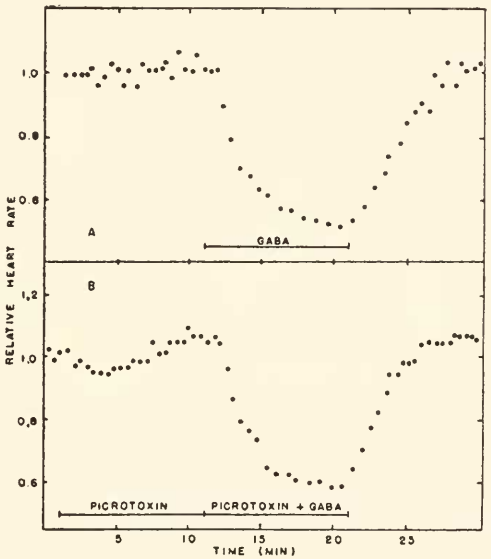

FIGURE 6. Rate changes in a heart perfused with GABA alone (2×10^{-5} *M*) and with GABA plus picrotoxin (1×10^{-3} *M*). See text for details.

in heart rate. The block of the inhibitor nerves, therefore, is not merely a reflection of the increased heart rate caused by the picrotoxin. The mean relative rate obtained by stimulation before treatment with the drug was 0.19 (SD = 0.15) *i.e.,* stimulation caused an 81% decrease in rate, while after picrotoxin treatment the mean relative rate was 0.76 (SD = 0.10). Thus, in the presence of the drug, stimulation decreased the rate by only 24%. A "*t*" test for the difference between these two means showed it to be significant ($P > 0.99$). In Figure 5 the mean relative rate produced by stimulation of the inhibitor nerves in the four hearts before, during and after perfusion with picrotoxin during each of the ten stimulation periods is shown. The mean decrease in rate produced by stimulation of the inhibitor nerves in the four hearts before picrotoxin perfusion was 20.2 beats per minute. During and after picrotoxin perfusion the decrease was 8.3 beats per

minute. We have no data concerning changes in contraction strength during stimulation of inhibitor nerves while perfusing with picrotoxin.

The reduced effectiveness of the inhibitor nerves outlasts the perfusion with picrotoxin. As can be seen from Figure 5 the mean relative rate obtained by stimulation 20 minutes after the end of perfusion with picrotoxin was still 0.80 (SD = 0.09), 0.60 unit greater than the mean relative rate obtained before picrotoxin treatment.

Since picrotoxin is effective in blocking the function of the cardioinhibitory nerves of *Limulus,* its ability to antagonize the action of applied GABA was also tested. Four isolated hearts were used in these experiments. Since from one preparation to the next there is considerable variation in the response to a given concentration of GABA, a control perfusion of 100 ml. of GABA was made for

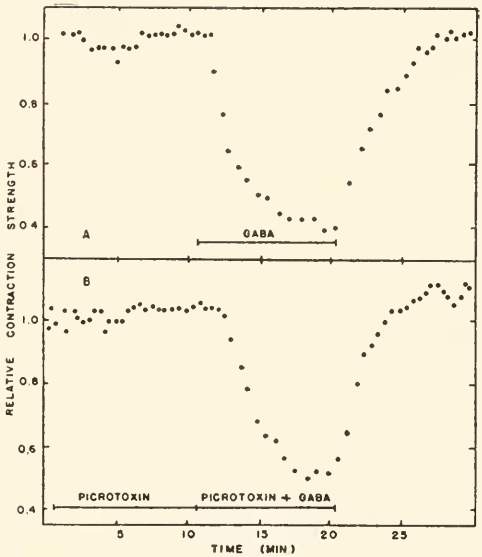

FIGURE 7. Contraction strength changes in a heart perfused with GABA alone (2×10^{-5} *M*) and with GABA plus picrotoxin (1×10^{-3} *M*). See text for details.

each heart prior to picrotoxin treatment. After the heart had recovered from the GABA perfusion by perfusing for one-half hour with drug-free saline, treatment with picrotoxin was begun. After perfusion with 100 ml. of 10^{-3} *M* picrotoxin in saline, a second 100-ml. portion containing the same concentration of GABA as that previously given was perfused. In this way GABA at concentrations of 5, 10 and 20 $\times$ 10^{-6} *M* was tested against picrotoxin at 10^{-3} *M*.

The response of the heart to GABA is not significantly altered by picrotoxin. Figure 6 presents the results for one of the four hearts. In the example shown the mean decrease in rate was 8.7 beats per minute during GABA perfusion prior to picrotoxin treatment. During GABA perfusion after picrotoxin treatment the mean decrease in rate was 7.7 beats per minute. Not only is the decrease in rate almost identical in the presence or absence of picrotoxin, but the time course of the response

to GABA is essentially unaltered. Although there were differences between relative rates obtained during GABA perfusion before and after picrotoxin treatment in individual hearts, the mean relative rate for the four hearts during GABA perfusion prior to picrotoxin treatment was 0.30, exactly the same as the mean value obtained during GABA perfusion after picrotoxin treatment (Mean Difference = 0.00; SD = 0.05).

In one of the hearts in which the interaction between GABA and picrotoxin was tested, data about strength changes were also obtained (Fig. 7). During perfusion with GABA alone the minimal relative contraction strength was 0.43. When GABA and picrotoxin were perfused together it was 0.50. The time course of the inhibition in both cases was approximately the same.

Discussion

We have considered the evidence that GABA acts as a synaptic transmitter in the cardioinhibitory pathway of *Limulus*. It is worthwhile comparing our observations with those on other arthropod systems in which GABA is believed to be a junctional transmitter.

In crustacean inhibitory motor neurons, GABA clearly appears to be the natural transmitter. It duplicates the effects of activation of the inhibitory neurons on muscle (Dudel, 1965; Takeuchi and Takeuchi, 1966), is present in the inhibitory axons and the synthetic machinery is present in such axons (Kravitz *et al.*, 1963).

Nearly as conclusive evidence exists that GABA is the natural transmitter for cardioinhibition in crustaceans. While it has not been isolated from this site, application to the ganglion cells of the crustacean heart has been shown to mimic, in all respects, the action of the natural transmitter (Florey, 1957; Maynard, 1961).

On the other hand, although the crustacean stretch receptor has been shown to be inhibited by GABA it does not appear to be the transmitter in this system (Kuffler and Edwards, 1958; Edwards and Kuffler, 1959).

Unequivocal proof that a given compound is the endogenous transmitter at a given junction is not easily obtained. Short of actual demonstration that the supposed transmitter is liberated by activity in the presynaptic fibers and that it, when applied in physiological concentrations, reproduces the conductance changes which occur during synaptic transmission (Terzuolo and Edwards, 1962), some doubt about the identity of the transmitter will exist. Because of the anatomical arrangement at many junctions it is difficult, if not impossible, to produce such direct evidence about the nature of the transmitter.

In view of this difficulty a number of other sets of criteria have been proposed which do not rely on such direct evidence. One such set is that of McLennan (1963): (1) The substance occurs in presynaptic structures. (2) An enzymatic mechanism for synthesis of the substance is present. (3) An enzyme system for inactivation of the substance is present. (4) Application of the substance mimics stimulation. (5) During stimulation the substance is detectable in perfusates. (6) Pharmacological agents which interfere with operation of the neuron similarly affect the action of the substance artificially applied.

If a given chemical is to be seriously considered to be the endogenous transmitter at a given junction then it must meet each of these criteria. Conversely, if a given chemical does not meet one or more of these criteria, it is doubtful that it is

the endogenous transmitter at that junction. Our evidence shows that GABA fails to meet two requirements. First, picrotoxin which effectively blocks the action of the inhibitor nerves is without effect upon the slowing of the heart rate caused by GABA (Criterion 6). Since the exact site of action of picrotoxin at the inhibitory junction is unknown, failure to meet this requirement alone is not sufficient to eliminate GABA as a possible transmitter at this junction. However GABA also fails to meet a second requirement, namely that it mimic stimulation of the inhibitor nerve (Criterion 4). Although stimulation and GABA both slow the heart rate, they have quite different effects upon the pattern of neural activity in the cardiac ganglion. Stimulation of the inhibitor nerves decreases the number of units discharging in the cardiac ganglion during each burst of electrical activity, as well as the total duration of each burst (Heinbecker, 1933). GABA produces neither of these changes in the pattern of the burst.

We believe, therefore, that even if GABA were to meet some of the other criteria listed above, it could not be seriously considered as a natural transmitter in the *Limulus* cardioinhibitory pathway.

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Summary

1. GABA (5×10^{-7} to 5×10^{-5} *M*) perfused through the isolated *Limulus* heart mimics stimulation of the cardioinhibitory nerves by decreasing rate and strength of beating of the heart.

2. GABA, unlike activity in the cardioinhibitory nerves, decreases neither the number of units discharging nor the total duration of each burst of electrical activity in the cardiac ganglion.

3. Picrotoxin (1×10^{-3} *M*) blocks the function of the cardioinhibitory nerves.

4. Picrotoxin (1×10^{-3} *M*) blocks neither the rate nor the strength-decreasing effects of applied GABA.

5. Since GABA does not mimic the action of the inhibitor nerves and its action is not blocked by an agent blocking the function of the inhibitor nerves, we believe it is probable that GABA is not a transmitter in the *Limulus* cardioinhibitory pathway.

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